Journal of Cell Science 110, 2547-2555 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JCS3585

Localisation of the *Schizosaccharomyces pombe* rho1p GTPase and its involvement in the organisation of the actin cytoskeleton

Manuel Arellano, Angel Duran and Pilar Perez*

Instituto de Microbiología Bioquímica, CSIC/Universidad de Salamanca, Edificio Departamental, 37007 Salamanca, Spain

*Author for correspondence (e-mail: piper@gugu.usal.es)

SUMMARY

The *Schizosaccharomyces pombe* rho1p GTPase directly activates the (1-3)β-D-glucan synthase and participates in the regulation of cell wall growth and morphogenesis in this fission yeast. Indirect immunofluorescence experiments using rho1p tagged with hemagglutinin have revealed that rho1p was located at the growing tips during interphase and at the septum prior to cytokinesis, localising to the same areas as actin patches. In *S. pombe cdc10-129* mutant cells, arrested in G_1, HA-rho1p accumulates at one tip whereas in *cdc25-22* mutants, arrested in G_2, HA-rho1p accumulates at both tips. In *tea1-1* and *tea2-1 cdc11-119* mutant cells, HA-rho1p is localised to the new growing tips. Overexpression of different *rho1* mutant alleles caused different effects on cortical actin patch distribution, (1-3)β-D-glucan synthase activation, and sensitivity to cell wall specific antifungal drugs. These results indicate that multiple cellular components are activated by rho1p. Overexpression of the dominant negative *rho1T20N* allele was lethal as was the *rho1*$^+$ deletion. Moreover, when *rho1*$^+$ expression was repressed in actively growing *S. pombe*, cells died in about 10 to 12 hours. Under these conditions, normal cell morphology was maintained but the level of (1-3)β-D-glucan synthase activity decreased and the actin patches disappeared. Most cells lysed after cytokinesis during the process of separation, and lysis was not prevented by an osmotic stabiliser. We conclude that rho1p localisation is restricted to growth areas and regulated during the cell cycle and that rho1p is involved in cell wall growth and actin cytoskeleton organisation in *S. pombe*.

Key words: GTPase, rho1p, Actin, Morphogenesis, Fission yeast

INTRODUCTION

The actin cytoskeleton plays an important role in maintaining cell morphology during the growth and cell division of yeast cells. The localisation of actin coincides with the sites of cell wall deposition throughout the cell cycle (Welch et al. 1994). In the budding yeast *S. cerevisiae*, the actin cytoskeleton assumes a polarised asymmetry and is involved in directing cell growth towards the bud. Cortical actin patches were observed to be associated with the cell surface via an invagination of plasma membrane (Mulholland et al., 1994), around which actin and associated proteins are organised. In the fission yeast *S. pombe*, cortical actin is associated with the deposition of cell wall material at the growing ends (Marks et al., 1986). During G_1 and S, growth occurs only at the old end, and cortical actin localises exclusively at this end. In early G_2, when the cells reach a certain size, growth begins also at the new end (new end take off or NETO) and becomes bi-directional (Mitchinson and Nurse, 1985), and actin is seen at both ends. At mitosis, cell growth ceases and actin patches disappear from the ends and reassemble at the medial region where the septum will be formed. After cytokinesis, actin moves to the old end where growth will be re-initiated (Marks and Hyams, 1985). A close relationship between actin reorganisation and cell wall formation was also observed in reverting *S. pombe* protoplasts where disruption of the actin cytoskeleton by cytochalasin B blocked protoplast regeneration and cell wall deposition (Kobori et al., 1989). The cortical actin cytoskeleton is therefore a very dynamic structure, and the changes in actin patches must be regulated spatially and temporally during the cell cycle.

The Rho family of GTPases are known to regulate actin cytoskeleton-dependent functions in animal cells (reviewed by Hall, 1994; Chant and Stowers, 1995; Ridley, 1995). In particular, RhoA regulates the formation of stress fibres and focal adhesions. Although the biochemical mechanism by which it causes these effects remains unknown, several downstream targets of Rho proteins have been identified recently that can potentially act on the actin cytoskeleton. For example, RhoA has been shown to physically interact with and activate a phosphatidylinositol-4 phosphate 5 kinase (PIP5K) (Ren et al., 1996). The product of this type of kinases, phosphatidylinositol 4,5-biphosphate (PIP2), regulates the interaction of profilin, α-actinin, and several actin-capping proteins, potentially leading to the assembly/disassembly of actin filaments (reviewed by Lee and Rhee, 1995). In addition, RhoA physically interacts with certain serine/threonine kinases such as PKN, rhophilin (Watanabe et al., 1996) and ROKα and ROKβ, which are homologous to the myotonic dystrophy kinase (Leung et al., 1995; Ishizaki et al., 1996). Finally, RhoA binds specifically to the myosin phosphatase that regulates the extent of phosphorylation of myosin light chain, and consequently the

interaction of actin and myosin leading to stress fibre formation (Kimura et al., 1996).

In *S. cerevisiae* Rho1p is essential (Madaule et al., 1987) and localises to the cell periphery at the site of bud growth. Cortical actin patches overlap the Rho1p staining sites (Yamochi et al., 1994). Rho1p has been suggested to be involved in the regulation of various enzymatic activities, but only Pkc1p (Nonaka et al., 1995) and (1-3)β-D-glucan synthase (Drgonová et al., 1996; Qadota et al., 1996) have been shown to be direct targets for this GTPase. A third target for Rho1p is Bni1p (Kohno et al., 1996), which shares homology with proteins involved in cytokinesis or the establishment of cell polarity and has been implicated in cytoskeletal control. Bni1p also interacts with the activated form of Cdc42p, with actin, and with two actin-associated proteins, profilin and Bud6p (Evangelista et al., 1997; Imamura et al., 1997).

In a previous study, we presented evidence that the *S. pombe* rho1p GTPase is directly involved in the specific regulation of (1-3)β-D-glucan synthase and the control of *S. pombe* morphogenesis (Arellano et al., 1996). Overexpression of the *rho1*$^+$ gene in wild-type *S. pombe* cells caused loss of polarity, and the expression of the constitutively active *rho1-G15V* mutant allele halted cell growth with aberrant morphology and a very thick cell wall. In the present study, we show the localisation of rho1p to the growing ends and the septation site of *S. pombe* in a position similar to that of the cortical actin patches. We also describe the effect of overexpressing different *rho1* alleles on actin patch localisation and how rho1p depletion causes the disappearance of cortical actin patches. These results suggest that rho1p is involved in the organisation of the actin cytoskeleton in *S. pombe*.

MATERIALS AND METHODS

Strains, growth conditions and genetic methods

Standard *S. pombe* media and genetic manipulations were employed (Moreno et al., 1991). All strains were isogenic to wild-type strains 972 h^- and 975 h^+. Yeasts were usually grown in YES medium (0.5% yeast extract, 3% glucose) or minimal medium (EMM) containing the required supplements. Incubations were carried out either at 32°C or at 37°C. Growth was monitored either by OD_{600} measurements or by cell number counting.

Escherichia coli DH5α was used as transformation host and for propagation of plasmids. *E. coli* CJ236 and MV1190 were used for the in vitro mutagenesis. They were grown in LB medium (1% Bactotryptone, 0.5% yeast extract, 1% NaCl) supplemented with 50 μg/ml ampicillin or 25 μg/ml kanamycin when appropriate.

Plasmids and recombinant DNA methods

The *S. pombe* plasmids pREP3X and pREP41X (Forsburg, 1993) allowing thiamine-regulated control of gene expression, were kindly provided by Dr S. Moreno. All DNA and RNA manipulations were carried out by established methods (Sambrook et al., 1989). Standard techniques for manipulation of fission yeast (Moreno et al., 1991) were used throughout. *S. pombe* was transformed by electroporation (Prentice, 1992) or by the lithium acetate method (Ito et al., 1983).

Site-directed mutagenesis was performed with the MUTAGENE kit (Bio-Rad) using the rho1+ cDNA cloned into the SalI and BamHI sites of Bluescript KS+ plasmid as the starting template. The G15V mutation was done as described previously (Arellano et al., 1996). For the V39T, F40Y, D121A, and T20N mutations we used the following oligonucleotides:

5′ CTATGTTCCCACTACTTTGAAAATTATG 3′, 5′ GTTCCCACTGTTT ATGAAAATTATGTAGC 3′,
5′ CCTATTTTGTTGCTTGTCTGTTGCAAGGCTGATC 3′, and 5′ GGTGCATGTGGTAAAAATTGCTTA TTAATTG 3′, respectively.

Two hemagglutinin (HA) epitopes were added at the 5′ end of *rho1+* open reading frame by subcloning the *rho1+* open reading frame into the *Nco*I-*Bam*HI sites of the pAS2 plasmid (kindly provided by S. J. Elledge).This plasmid contains two HA epitopes in frame with the *Nco*I site. An *Eco*RI-*Bam*HI fragment containing the HA-rho1p was then subcloned into Bluescript SK+, and finally was subcloned as a *Sal*I-*Bam*HI fragment into the *Sal*I-*Bam*HI sites of pREP3X.

rho1 gene disruption

A deletion mutant allele of the *rho1*$^+$ gene was constructed by replacement of the whole open reading frame with the *ura4*$^+$ gene. 1 kb of the *rho1*$^+$ gene 5′ flanking region was amplified by PCR using primers with appropriate restriction sites (*Sal*I-*Pst*I) and cloned directly into the pGEMT vector (Promega). A *Sal*I-*Pst*I fragment from this plasmid was introduced into a different pGEMT-based plasmid with a *Bam*HI site (pGEMT-NRHO1). A *Pst*I-*Bam*HI fragment containing the *ura4*$^+$ gene was cloned into the same restriction sites of pGEMT-NRHO1 to give pGEMT-NURA4. Finally 1.3 kb of the *rho1*$^+$ 3′ flanking region was amplified by PCR using primers with appropriate restriction sites and cloned into the *Bam*HI-*Sph*I sites from the pGEMT-NURA plasmid to give the pGEMΔRHO1 plasmid. A 4.3 kb fragment from this plasmid containing the *ura4*$^+$ gene flanked by the 5′ and 3′ regions of the rho1+ gene, was used to transform a diploid strain (h^+ *ura4*-D18 *leu1-32 ade6*-M216/h^- *ura4*-D18 *leu1-32 ade6*-M210). Stable *ura*$^+$ transformants were obtained, and PCR analysis of genomic DNA confirmed the *rho1*$^+$ deletion.

HA-rho1$^+$ replacement

The *ura4*$^+$ gene from the pGEMΔRHO1 plasmid used for the disruption was replaced by the HA-tagged *rho1*$^+$ open reading frame as follows: the plasmid was cut with *Nco*I-*Bam*HI. The *Nco*I site was present in the reverse primer used to amplify the 5′ region of the *rho1*$^+$ gene by PCR. A fragment *Eco*RI-*Bam*HI containing HA-*rho1*$^+$ was obtained from the pAS2 plasmid where *rho1*$^+$ was cloned before. The *Nco*I and *Eco*RI overhangs were refilled with Klenow before the HA-*rho1*$^+$ ligation to the plasmid. A 3.2 kb fragment containing the HA-*rho1*$^+$ gene was used to transform a haploid strain (*ade6*-M210, *leu1-32, ura4*-D18, *rho1::ura4*$^+$) carrying the plasmid pREP41X-RHO1. Colonies able to survive in rich medium and auxotrophic for uracil were selected, and PCR analysis confirmed the replacement; this strain was designated MA1.

Microscopy techniques

For tubulin and actin staining, cells were fixed in methanol for at least 15 minutes. Immunofluorescence was performed as described (Hagan and Hyams, 1988). The primary antibody for microtubule staining was the monoclonal TAT1, kindly provided by K. Gull. For actin staining, the primary antibody was the monoclonal N350 (Amersham Corp.). For anti-hemaglutinin staining, the monoclonal antibody 12CA5 was used (BABCO). The secondary antibody in all cases was a sheep anti-mouse Cy3-conjugated F(ab′)2 fragment (Sigma Chemical Co). Actin staining was also performed with rhodamine-phalloidin as described (Chang et al., 1996). For double staining actin/HA, a rabbit polyclonal anti-hemaglutinin antiserum (BABCO) was biotinylated as previously described (Harlow and Lane, 1988) and used at 1:250 dilution. This antibody was visualised using 1:100 diluted FITC-streptavidin (Sigma). Cells were immobilised on poly-L-lysine coverslips and observed using a Zeiss Axiophot microscope or photographed using a confocal microscope (MRC600; Bio-Rad) with a 0.2 μm interval between successive pictures and analysed using Bio-Rad software.

Western blotting

HA-rho1p expressed in *S. pombe* cells was detected by western blot.

About 1×10^8 exponentially growing cells in minimal medium with or without thiamine were harvested by brief centrifugation, washed once in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5, containing 100 μM *p*-aminophenyl methanesulphonyl fluoride, leupeptin, and aprotinin) and resuspended in 100 μl of the same buffer. About 1 g of pre-chilled glass beads were added to the cells, and lysis was achieved in a Fast-Prep centrifuge (Bio 101 Savant) using two 15 second intervals at 5.5 speed. The resulting homogenates were collected, and glass beads and large debris were removed by centrifugation for 5 minutes at 450 ***g***. A sample was diluted 2× with lysis buffer containing 2% Triton X-100. The rest of the homogenate was centrifuged for 1 hour at 50,000 ***g***. The pellet was rinsed in lysis buffer and resuspended in lysis buffer with 1% Triton X-100. Total lysate, cytosolic and membrane fractions were subjected to SDS-PAGE, and separated proteins were electrophoretically transfered to a nitrocellulose membrane sheet (Schleicher and Schuell, Keene, NH). The blot was processed to detect HA-rho1p with 1:2,000 diluted 12C5A mAb as primary antibody and the ECL detection kit (Amersham Corp.).

Measurement of (1-3)β-D-glucan synthase activity

Cell-free extracts were prepared and (1-3)β-D-glucan synthase activities assayed as described previously (Ribas et al., 1991) with some modifications. Basically, 3×10^9 cells were harvested, washed with 1 mM EDTA, and resuspended in 300 μl of the same buffer. Lysis was achieved in a Fast-Prep centrifuge using 0.5 g of glass beads and spinning twice for 20 seconds at 6.0 speed. The resulting homogenates were collected adding 30 ml of 50 mM Tris-HCl, pH 8.0, in 1 mM EDTA. The cell walls were removed by centrifugation for 5 minutes at 450 ***g***. The supernatant was centrifuged for 30 minutes at 50,000 ***g***, and the membrane pellet was resuspended in 0.5 ml buffer containing 50 mM Tris-HCl, 1 mM EDTA, 1 mM β-mercapto-ethanol, and 30% glycerol. Protein was determined by the Lowry method. A unit of activity was considered to be the amount that catalyses the incorporation of 1 μmol of substrate (UDP-D-glucose) per minutes at 30°C.

Labelling and fractionation of cell wall polysaccharides

Exponentially growing cultures of *S. pombe* wild type and transformants were supplemented with [U-^{14}C]glucose (1 μCi/ml) and incubated for an additional 4 hours. Cells were harvested and unlabelled cells were added to the radioactive samples as carriers. Total glucose incorporation was monitored by measuring the radioactivity in trichloroacetic acid insoluble material. Mechanical breakage of cells was done as described for the measurement of the (1-3)β-D-glucan synthase activity. Cell walls were pelleted at 1,000 ***g*** for 5 minutes and washed with 5% NaCl three times and with EDTA 1 mM three times. 100 μl aliquots of the total wall were incubated with 100 units of zymolyase 100T (Seikagaku Kogyo Co. Ltd) or Quantazyme (Quantum Biotechnologies Inc.) for 36 hours at 30°C. Aliquots without enzymes were included as control. The samples were centrifuged and the supernatant and washed pellet were counted separately. The supernatants from the zymolase 100T reaction were considered to be β-glucan plus galactomannan, and the pellet α-glucan. The supernatants from the Quantazyme reactions were considered to be β-glucan and the pellet α-glucan plus galactomannan.

RESULTS

rho1p is localised to the growing tips and the septum

In order to localise the rho1p GTPase in *S. pombe* cells, we added two hemagglutinin (HA) epitopes to the N terminus of the *rho1*$^+$ gene. The HA-tagged *rho1*+ gene integrated under its own promoter HA-*rho1*$^+$ was able to rescue the *rho1*$^+$ deletion lethal phenotype (data not shown). HA-*rho1*$^+$ was introduced into the pREP3X plasmid under the control of the *nmt1* promoter. Overexpression of HA-*rho1*$^+$ increased the β (1,3) glucan synthase activity as overexpression of *rho1*$^+$ gene (see Table 1).

Table 1. (1-3)β-D-glucan synthase activity in *S. pombe leu1-32* transformed with different plasmids

	Specific activity* (%)		
	30°C		37°C
Plasmid	+GTP	–GTP	+GTP
pREP3X	11.5±0.4 (100)	1.1	10.8±1.1 (100)
pREPRHO1	18.4±0.9 (160)	1.2	13.5±1.3 (125)
pREPHA-RHO1	17.9±1.4 (148)	ND	ND
pREPRHO1-G15V	41.2±6.1 (358)	41.8	41.6± 5.1 (385)
pREPRHO1-V39T	8.6±0.6 (75)	0.9	6.8±0.6 (63)
pREPRHO1-F40Y	7.8±0.7 (68)	1.0	4.1±0.5 (38)
pREPRHO1-D121A	13.3±1.6 (116)	1.5	9.1±1.0 (84)
pREPRHO1-T20N	7.8±0.8 (68)	ND	ND

All extracts were prepared from cells grown at 32°C in minimal medium without thiamine for 20 hours, except for the cells transformed with plasmid pREPRHO1-T20N, which were grown for 14 hours.

*Expressed as milliunits per mg of protein. Values are the means and standard deviations calculated from at least three independent experiments.

ND, not determined.

S. pombe leu1-32 cells transformed with this plasmid (pREPHA-RHO1) were grown in minimal medium without thiamine. The kinetics of HA-rho1p production were analysed by western blotting (Fig. 1). In the presence of thiamine, HA-rho1p was undetectable in total cell extracts. After 12 hours of induction, the level of HA-rho1p was slightly higher than in a *S. pombe* strain (MA1) carrying the HA-tagged *rho1*$^+$ gene integrated under its own promoter (see Fig. 1). The level of HA-rho1p increased with time of induction until 18 hours. Fractionation of the extracts revealed the presence of HA-rho1p in both soluble and membrane fractions. Some protein was visualised in the membrane fraction even in the presence of thiamine, because

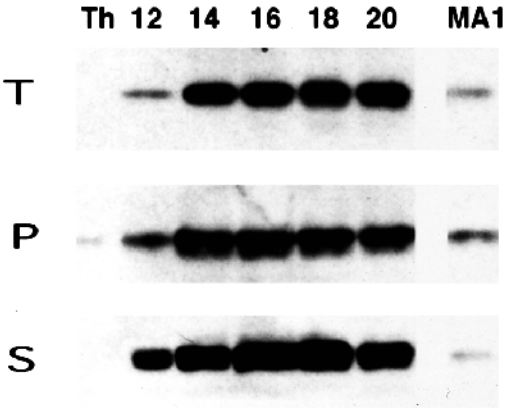

Fig. 1. Kinetics of HA-Rho1p expression upon induction of the *nmt1* promoter in plasmid pREPHA-RHO1. Western blotting was performed using 10 μg of protein per lane (or 20 μg for the strain MA1). The anti-HA monoclonal antibody 12CA5 was used at 1:2,000 dilution. Cells growing in mimimal medium with thiamine (Th) were washed three times and then grown in mimimal medium for 12, 14, 16, 18, and 20 hours. Strain MA1, with HA-*rho1*$^+$ integrated under its own promoter (see Materials and Methods), was used as control. T, total extract; P, membrane proteins; S, cytoplasmic proteins.

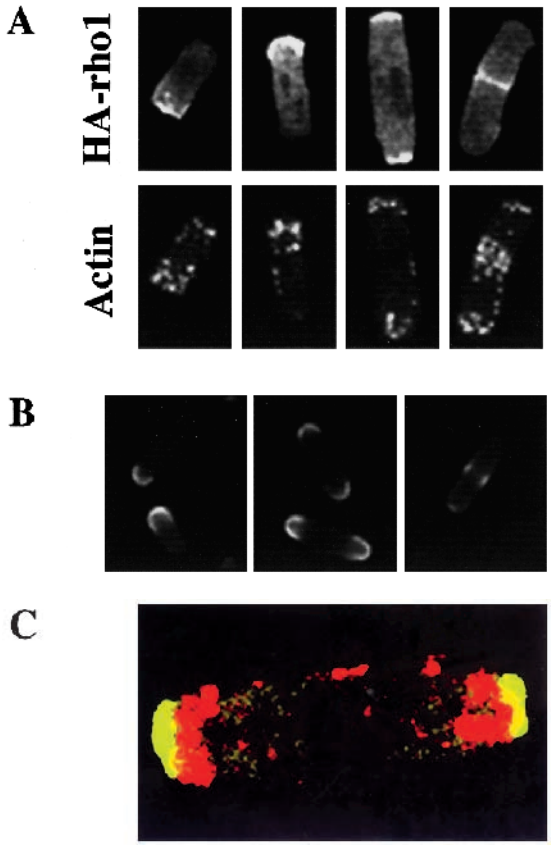

Fig. 2. Distribution of actin and HA-rho1p at different stages of the cell cycle. (A) Cells were double stained with the monoclonal antibody N350 (top panels) or biotinylated rabbit polyclonal anti-HA (lower panels). Medial staining after cytokinesis (left panels). Unipolar and bipolar staining during interphase (central panels) and medial staining in cells progressing through septation (right panels). (B) Optical sections obtained by confocal microscopy of cells stained with the anti-HA monoclonal antibody 12CA5. (C) The overlaid staining is shown with HA-rho1p in green and actin in red.

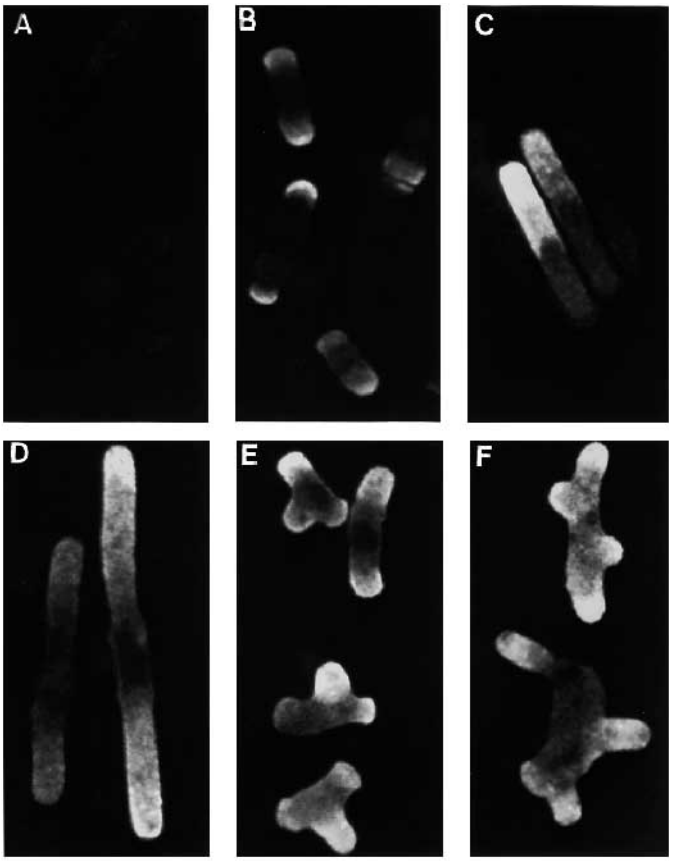

Fig. 3. Fluorescence micrographs of different *S. pombe* strains transformed with plasmid (A) pREPRHO1P or (B-F) pREPHA-RHO1P and grown in minimal medium without thiamine for 12 hours at 32°C and then for 3 hours at 37°C. (A and B) wild type; (C) *cdc10-129*; (D) *cdc25-22*; (E) *tea1-1*; (F) *tea2-1, cdc11-119*. The cells were fixed and stained with the anti-HA monoclonal antibody 12CA5.

the *nmt1*$^+$ promoter is slightly leaky under repressing conditions. HA-rho1p was not clearly detected by immunofluorescence in strain MA1 nor in the pREPHA-RHO1P transformant after 12 hours of induction. After 14 to 16 hours of induction, HA-rho1p was overproduced, but cell polarity was still normal. Therefore, cells were fixed and stained with 12CA5 anti-HA antibody and antibodies against actin, at this time.

The distribution of cortical actin patches at different stages of the cell cycle is well characterised in *S. pombe* (Snell and Nurse, 1993). HA-rho1p was found to be localised to the same regions as were the cortical actin patches (Fig. 2A and C). In interphase cells, both actin patches and HA-rho1p appeared at the cell tips whereas during mitosis they concentrated in the medial region where the septum will later be formed. After cell separation, HA-rho1p and actin patches colocalised to the growing old end. Observation of confocal sections suggested staining of the plasma membrane, in agreement with the western blot results (Fig. 2B).

To further characterise the cell-cycle-regulated presence of rho1p at the growing tips, HA-rho1p was expressed in the cell cycle thermosensitive mutants *cdc10-129* and *cdc25-22*. When cultured at 37°C, *cdc10-129* cells arrest in G_1, and cell growth is polarised to one of the tips, whereas *cdc25-22* cells arrest at the end of G_2 with both ends of the cell actively growing (Nurse et al., 1976). pREPHA-RHO1 was transformed into these cells and induced for 12 hours. Then the cultures were incubated for 3 to 4 hours at the restrictive temperature, and cells were stained with mAb 12C5A. HA-rho1p protein accumulated at the unique growing tip in *cdc10-129* cells whereas in *cdc25-22* mutant cells, HA-rho1p appeared at both tips (Fig. 3C,D). In both cases, staining spread away from the tips in the elongated cells.

We also examined the localisation of HA-rho1p in two polarity mutants transformed with pREPHA-RHO1. The *tea1-1* mutant cells mislocalise one of the two growing poles and become branched, and the double mutant *tea2-1 cdc11-119* activates new growing ends (Verde et al., 1995). In both mutants, HA-rho1p localised to the new growing tips (Fig. 3E,F). These results indicate that the genes affected in *tea1-1* and *tea2-1* are upstream of *rho1*$^+$ in the control of polarity and determine its localisation.

Overexpression of different rho1 alleles causes different effects on actin localisation, (1,3)-β-D-glucan-synthase activation, and antifungal drug sensitivity

It was reported previously that overexpression of the *rho1*$^+$

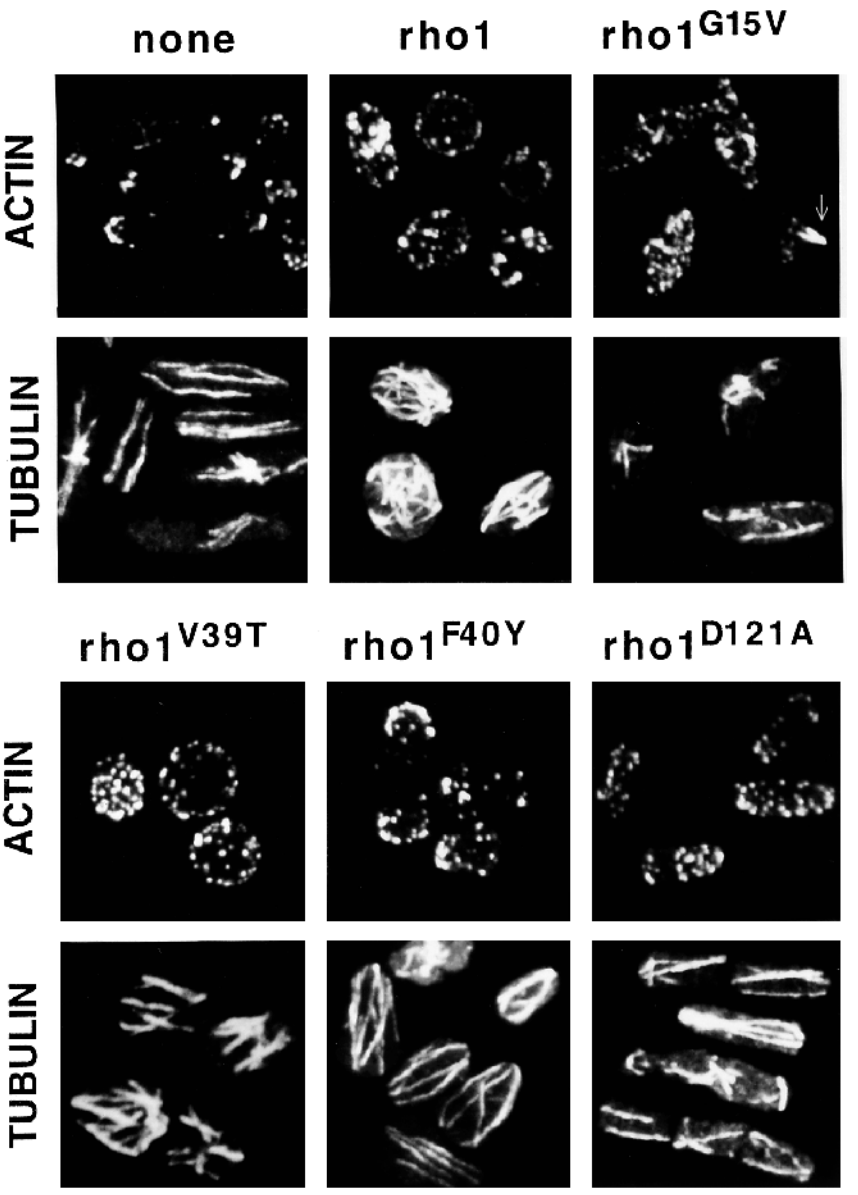

Fig. 4. Actin and tubulin organization in *S. pombe* cells overexpressing the indicated *rho1* alleles. Cells were fixed after growing for 20 hours in minimal medium without thiamine at 32°C.

gene in *S. pombe* causes loss of polarity and the cells become rounded (Arellano et al., 1996). To investigate the possible relationship between the rho1p GTPase and the actin cytoskeleton in *S. pombe*, we analyzed the actin patch distribution when several different *rho1* alleles were overexpressed. The *rho1-V39T* and *rho1-F40Y* alleles have thermosensitive mutations in the proposed rho1p effector domain (Nonaka et al., 1995). The mutation equivalent to D121A was originally described as an activating mutation in H-Ras, but it behaves as a dominant negative mutation in Cdc42p (Miller and Johnson, 1994). *rho1-G15V* is a constitutively active allele. As shown in Fig. 4, overexpression of *rho1*$^+$ or *rho1-V39T* made cells rounded and caused actin mislocalisation. *rho1-F40Y* altered the actin distribution only slightly, and cells were shorter than the wild-type but kept a cylindrical shape. Interestingly, *rho1-D121A* caused some actin mislocalisation although the cells were elongated and multiseptated instead of round. Overexpression of the constitutively active allele *rho1-G15V* is lethal (Arellano et al., 1996) and caused actin depolarisation with the formation of strongly stained cable-like actin structures (see arrow, Fig. 4).

The distribution of microtubules was also examined in cells expressing the different *rho1* alleles. Cytoplasmic microtubules appeared normal in all cases except for the *rho1-G15V* transformant in which they appeared aberrant (Fig. 4). Calcofluor staining of the cell wall did not show significant differences for the cells overexpressing *rho1-V39T rho1-F40Y* or *rho1-D121A* as compared with wild type (data not shown).

We also determined the effects of the different *rho1* alleles on the known target (1-3)β-D-glucan synthase. Only cells overexpressing *rho1* or *rho1G15V* have this enzyme activated (Table 1). *rho1-V39T* and *rho1-F40Y* caused a small but significant decrease (25 and 32%, respectively) in the activity, and *rho1-D121A* essentially did not alter the (1-3)β-D-glucan synthase levels. When the activity was measured at 37°C, the decrease caused by overexpressing the *rho1-F40Y* allele was more pronounced (66%). We also transformed *S. pombe* cells with the *rho1-T20N* dominant negative allele. Overexpression

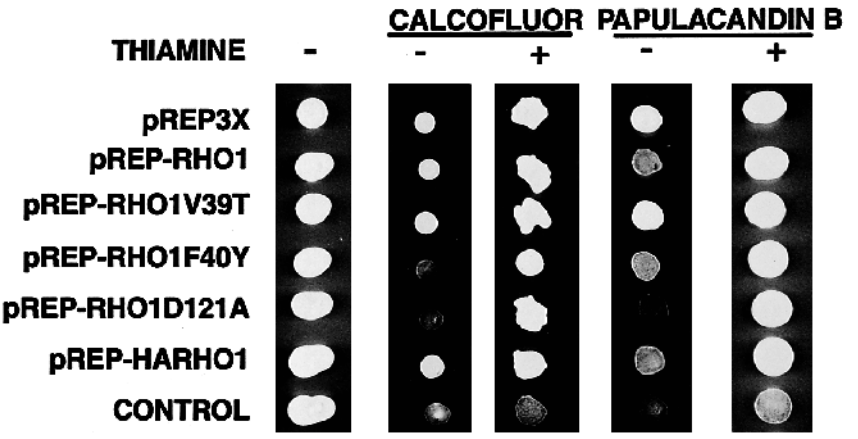

Fig. 5. Sensitivity to Calcofluor (0.5 mg/ml) or Papulacandin B (2.5 μg/ml) of *S. pombe* cells overexpressing different *rho1* alleles. The control is a mutant strain hypersensitive to Calcofluor and Papulacandin B (kindly provided by Dr Y. Sanchez).

of this allele caused a drastic lysis of the cells; therefore, we could not analyse the effects of this allele on actin localisation. The (1-3)β-D-glucan synthase activity in cells expressing this allele was measured after 14 hours of induction from the *nmt1* promoter, when there is only 5-10% lysis of the cells and enzyme activity was already considerably decreased (32%).

The resistance to antifungal drugs affecting the cell wall was determined for all of the transformants except for those carrying *rho1-G15V* and *rho1-T20N*, which were lethal. As described previously (Arellano et al., 1996), *rho1*$^+$ overexpression caused hypersensitivity to Papulacandin B (Fig. 5). *rho1-V39T* did not caused any changes with respect to sensitivity. By contrast, overexpression of *rho1-F40Y* or *rho1-D121A* caused a marked hypersensitivity to both Calcofluor and Papulacandin B.

Cell wall composition of the transformants grown during 22 hours in the absence of thiamine was analysed (Table 2). Incorporation of radiactive glucose into the cell wall with respect to total radiactivity in cells overexpressing the *rho1-V39T* allele was similar to that of the pREP3X transformant whereas in *rho1-F40Y* overexpressing cells was lower (77% as compared with the pREP3X transformant). These results could explain the hypersensitivity of *rho1-F40Y* transformant to drugs affecting the cell wall. No significant differences in cell wall composition were detected in these transformants.

Cells overexpressing *rho1-D121A* have a slightly higher level of glucose incorporation into the cell wall. A significant increase in β-glucan percentage was observed with a concomitant decrease in galactomanan. This altered wall composition was previously observed in *rho1-G15V* transformants and might be the cause of the hypersensibility to drugs affecting the cell wall in this transformant.

rho1$^+$ is an essential gene and rho1p depletion causes cell lysis and actin depolymerisation

In order to investigate further the function of *rho1*$^+$, we deleted this gene, replacing its open reading frame with the *ura4*$^+$ gene. A diploid strain was transformed, and stable uracil-prototrophic colonies were isolated containing one disrupted allele and one wild-type copy of *rho1*$^+$, as confirmed by PCR analysis. Sporulation of this transformant was induced, and tetrads were dissected. All tetrads resulted in only two viable *ura*$^-$ spores (data not shown); therefore, *S. pombe rho1*$^+$ is essential for vegetative growth. To further explore the function of *rho1*$^+$, the heterozygous diploid was transformed with the plasmid pREP41XRHO1, which carries the *rho1*$^+$ gene under the control of a reduced expression rate *nmt* promoter (Forsburg, 1993) and the *LEU2* gene. *leu*$^+$ *ura*$^+$ *ade*$^-$ haploid segregants with the plasmid grew on adenine-supplemented minimal medium without thiamine and exhibited wild-type morphology. Repression of *rho1*$^+$ expression by adding thiamine to the culture medium resulted in cell lysis after 10 to 12 hours, depending on the incubation temperature. Lysis occurred mainly after cytokinesis (Fig. 6B). Flow cytometry analysis was performed to determine the DNA contents of cells at different times after repression of the *nmt* promoter with thiamine. Cells always showed the 2C peak as observed in wild-type *S. pombe* (data not shown).

During repression of the *nmt* promoter, the (1-3)β-D-glucan synthase activity decreased as shown in Fig. 6A. In the absence of thiamine, the activity was twice that of a wild-type strain whereas 9 hours after repression, when around 15% of the cells were lysed, the activity was approximately one third that of the wild type. No morphological abnormalities such as cell swelling were observed, and medium supplemented with 1.2 M sorbitol did not protect cells from death.

Table 2. Incorporation of radiactivity from [^{14}C]glucose into cell wall polysaccharides of different *S. pombe* transformants grown without thiamine for 22 hours

Plasmid	Cell wall	Galactomannan	α-Glucan	β-Glucan
pREP3X	29.6±1.7	4.7±1.3 (15.8)	9.8±0.9 (33.0)	14.3±1.5 (50.0)
pREPRHO1V39T	31.7±1.6	4.3±0.7 (13.5)	10.8±0.6 (34.2)	15.9±1.4 (50.2)
pREPRHO1F40Y	22.9±1.8	3.7±0.9 (13.7)	6.7±0.6 (29.0)	12.1±1.2 (52.6)
pREPRHO1D121A	34.0±0.5	2.1±0.5 (6.1)	11.6±0.7 (34.2)	20.4±1.6 (60.1)

Values indicate percentage of total [^{14}C]glucose incorporated and are the means ± s.d. from at least three independent experiments. Values in parentheses are percentages of the corresponding polysaccharides in the cell wall.

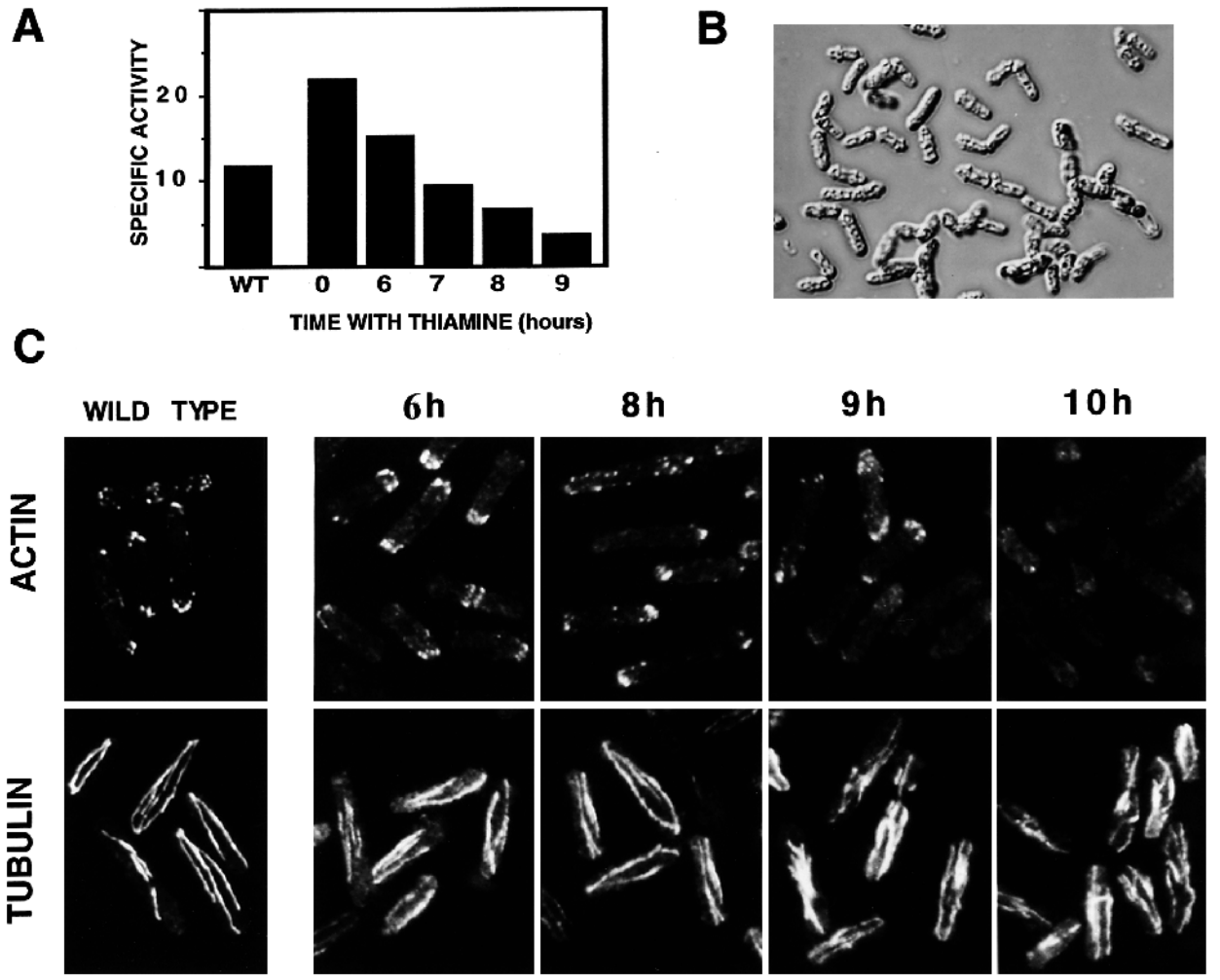

Fig. 6. (A) (1-3)β-D-glucan synthase activity in *S. pombe leu1-32, ade6-M210, rho1::ura4*$^+$ transformed with pREP41XRHO1. All extracts were prepared from cells grown at 32°C in minimal medium without thiamine and switched to medium with thiamine for the indicated times. (B) Lysis of the cells after 12 hours with thiamine. (C) Actin and tubulin staining of the rho1p-depleted cells. At the indicated times after switching to medium with thiamine, cells were fixed and stained with the monoclonal antibodies N350 or TAT1, respectively.

The pREP41XRHO1-transformed cells were stained to visualise actin and tubulin while growing without thiamine or at different times after the addition of thiamine to the medium. Staining with the N350 and TAT1 antibodies (Fig. 6C) showed that after 8 to 10 hours, when most of the cells have not yet undergone lysis, the actin patches had largely disappeared while the microtubules remained intact. Actin staining using rhodamine-phalloidin gave the same result. We could not see differences in the total actin amount of cell extracts obtained at different times after the addition of thiamine, as detected by western blot (data not shown). It seems, therefore, that *S. pombe* rho1p GTPase is required for formation or maintenance of the cortical actin patches.

DISCUSSION

Much work in recent years has concerned how yeast cells become polarised and divide in precisely defined spatial patterns. Fission yeast cells have a cylindrical shape, maintain a constant diameter by growing and elongating at the tips, and then divide by fission forming a septum in the medial region. Among the proteins involved in the control of morphogenesis, GTPases play a central role. In budding yeast, the Rsr1p/Bud1p, Cdc42p and Rho1p GTPases are all involved in the development of cell polarity and may function in a signalling cascade (Chant and Stowers, 1995). In fission yeast, Ras1p, Cdc42p and the Rho proteins may have similar roles and functional relationships.

Use the epitope tagging has enabled us to determine the intracellular localisation of rho1p to sites of cell growth, both in wild-type cells and in the polarity mutants *tea1-1* and *tea2-1 cdc11-119*. We conclude that rho1p must act downstream of the proteins encoded by *tea1* and *tea2* in establishing polarised cell growth. This is consistent with the proposal that tea1p and tea2p function as positional markers required to establish correct spatial organisation, similar to Bud3p and Bud4p in *S. cerevisiae* (Verde et al., 1995). tea1p has been recently cloned and shown to be located at the cell poles (Mata and Nurse, 1997).

Different *rho1* mutant alleles were overexpressed in an attempt to separate the positive rho1p activity on the (1,3)β-D-

glucan-synthase from other rho1p effects such as growth depolarisation and actin mislocalisation. Alleles carrying the V39T and F40Y mutations in the effector domain are clearly impaired in activation of the (1,3)β-D-glucan-synthase because this activity is lower in cells overexpressing these *rho1* alleles compared with those overexpressing *rho1*$^+$. However, *rho1-V39T* overexpression still causes a defect in actin localisation and cell polarisation similar to that caused by *rho1*$^+$, whereas *rho1-F40Y* is less effective in depolarisation but causes a more severe cell wall defect that is reflected in the decrease of (1,3)β-D-glucan-synthase activity at 37°C and in hypersensitivity to Calcofluor and Papulacandin B, probably due to a decrease in the total amount of cell wall. These results suggest that rho1p interaction with the (1,3)β-D-glucan-synthase activity is independent of the rho1p effects on actin localisation. The results obtained overexpressing the *rho1-D121A* allele also support this hypothesis since this allele has no effect on (1,3)β D-glucan-synthase activity but causes a severe morphological phenotype with large elongated and multiseptated cells. These cells have an altered cell wall composition, are hypersensitive to Calcofluor and Papulacandin B and the actin patches are mislocalised although the cells are elongated and keep cylindrical shape.

Pkc1p and Bni1p are known Rho1p targets in *S. cerevisiae* (Nonaka et al., 1995; Kohno et al., 1996). The rho1p effect on *S. pombe* actin could be mediated by the product of *pck2*$^+$, one of the genes encoding PKC enzymes in *S. pombe* (Toda et al., 1993). pck2p has effects on actin and cell wall integrity (Kobori et al., 1994), but further studies will be required to know if pck2p directly interacts with rho1p. Another possible target of rho1p that can affect actin organisation in *S. pombe* is cdc12p (Chang et al., 1997). This protein, recently described, is required for cytokinesis, interacts with profilin and belongs to the Bni1p family of proteins.

rho1p depletion causes cell death concomitant with a decrease in (1,3)β-D-glucan-synthase activity. Cell lysis occurs mainly after cytokinesis, probably because correct cell wall formation is essential at that point of the cell cycle. However, lysis is not prevented by an osmotic stabiliser; therefore, rho1p probably has other effects besides the activation of (1,3)β-D-glucan-synthase that are essential to the cell. rho1p depletion did not cause cells to arrest in the G_1 stage of the cell cycle, as has been described in animal cells (Yamamoto et al., 1993). A clear effect of rho1p depletion in *S. pombe* seems to be disassembly of actin patches. This phenomenon has already been described in animal cells (Chardin et al., 1989), where disappearance of actin microfilaments was observed upon inactivation of Rho using the *Clostridium botulinum* C3 toxin. Perhaps actin patch disassembly is one of the lethal effects of *S. pombe* rho1p depletion, because in *S. cerevisiae* actin patches are essential for viability (Adams et al., 1993; Moon et al., 1993).

The finding that cell polarity is maintained upon rho1p depletion is important. It appears that rho1p acts downstream of other proteins that define polarity, and some of these proteins may direct rho1p to the growth areas where cortical actin patches also localise. Understanding the mechanisms that specify the position of actin patches is crucial to understanding polarised cell growth. Previous studies have indicated that cell cycle regulators specify the localisation of actin at different stages of the cell cycle (Lew and Reed, 1995). rho1p localises to the same places as the actin patches during the cell cycle in *S. pombe* and the results shown in this study using the *cdc10-109* and *cdc25-22* mutants suggest that rho1p position is also cell cycle regulated. It is possible that rho1p is the final component in a GTPase cascade linking the cell cycle controls to cell wall biosynthesis. Thus, rho1p might act directly on the (1,3)β-D-glucan synthase and also be required for actin patch formation in the growth areas where they will be necessary for secretion and cell wall formation (Kobori et al. 1991). Although preliminary, our data support this hypothesis. Thus, when rho1p is overproduced, it does not localise to the growth areas nor do the actin patches, and the cells lose polarity. The same phenotype occurs when proteins upstream of rho1p in the control of polarity, such as cdc42p or ras, are overproduced. On the other hand, when rho1p is depleted, cortical actin patches disappear. Identification of rho1p downstream targets other than the (1,3)β-D-glucan synthase in *S. pombe* will be necessary to understand how rho1p regulates cytoskeletal organisation in this yeast.

We thank Drs S. Moreno, C. Roncero, Y. Sanchez, J. C. Ribas, and H. Valdivieso for a critical reading of the manuscript. We thank Dr P. Nurse for the generous gift of strains and antibodies and for helpful comments on the manuscript and P. Jordan, J. Mata and other members of the ICRF Cell Cycle Group for assistance with the confocal microscopy and image processing. We thank C. Belinchon for technical help. M. Arellano acknowledges support from fellowship granted by the Universidad de Salamanca, Spain. This work was supported by grant BIO95-0500 from the Comisión Interministerial de Ciencia y Tecnología, Spain and by a contract with the company Lilly S.A., Spain.

REFERENCES

Adams, A. E. M., Cooper, J. A. and Drubin, D. G. (1993). Unexpected combinations of null mutations in genes encoding the actin cytoskeleton are lethal in yeast. *Mol. Biol. Cell* **4**, 459-468.

Arellano, M., Durán, A. and Perez, P. (1996). Rho1p GTPase activates (1-3)β-D-glucan synthase and is involved in *Schizosaccharomyces pombe* morphogenesis. *EMBO J*. **15**, 4584-4591.

Chang, F., Woollard, A. and Nurse, P. (1996). Isolation and characterization of fission yeast mutants defective in the assembly and placement of the contractile actin ring. *J. Cell Sci*. **109**, 131-142.

Chang, F., Drubin, D. and Nurse, P. (1997). Cdc12p, a protein required for cytokinesis in fission yeast, is a component of the cell division ring and interacts with profilin. *J. Cell Biol*. **137**, 169-182.

Chant, J. and Stowers, L. (1995). GTPase cascades choreographing cellular behavior: movement, morphogenesis, and more. *Cell* **81**, 1-4.

Chardin, P., Boquet, P., Madaule, P., Popoff, M. R., Rubin, E. J. and Gill, D. M. (1989). The mammalian G protein rhoC is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilaments in Vero cells. *EMBO J.* **8**, 1087-1092.

Drgonová, J., Drgon, T., Tanaka, K., Kollár, R., Chen, G.-C., Ford, R. A., Chan, C. S. M., Takai, Y. and Cabib, E. (1996). Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. *Science* **272**, 277-279.

Evangelista, M., Blundell, K., Longtine, M. S., Chow, C. J., Adames, N., Pringle, J. R., Peter, M. and Boone, C. (1997). Bni1p, a yeast formin linking Cdc42p and the actin cytoskeleton during polarized morphogenesis. *Science* **276**, 118-121.

Forsburg, S. L. (1993). Comparison of *S. pombe* expression systems. *Nucl. Acids Res*. **21**, 2955-2956.

Hall, A. (1994). Small GTP-binding proteins and the regulation of the actin cytoskeleton. *Annu. Rev. Cell Biol.* **10**, 31-54.

Hagan, I. M. and Hyams, J. S. (1988). The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci*. **89**, 343-357.

Harlow, E. and Lane, D. (1988). *Antibodies. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.

Imamura, H., Tanaka, K., Hihara, T., Umikawa, M., Kamei, T., Takahashi, K., Sasaki, T. and Takai, Y. (1997). Bni1p and Bnr1p: downstream targets of the Rho family small G-proteins which interact with profilin and regulate actin cytoskeleton in *Saccharomyces cerevisiae*. *EMBO J.* **16**, 2745-2755.

Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N. and Narumiya, S. (1996). The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO J.* **15**, 1885-1893.

Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol*. **153**, 163-168.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* **273**, 245-248.

Kobori, H., Yamada, N., Taki, A. and Osumi, M. (1989). Actin is associated with the formation of the cell wall in reverting protoplasts of the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **94**, 635-646.

Kobori, H., Toda, T., Yaguchi, H., Yanagida, M. and Osumi, M. (1994). Fission yeast protein kinase C gene homologues are required for protoplast regeneration: a functional link between cell wall formation and cell shape control. *J. Cell Sci.* **107**, 1131-1136.

Kohno, H., Tanaka, K., Mino, A., Umikawa, M., Imamura, H., Fujiwara, T., Fujita, Y., Hotta, K., Qadota, H., Watanabe, T., Oyha, Y. and Takai, Y. (1996). Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP binding protein in *Saccharomyces cerevisiae*. *EMBO J.* **15**, 6060-6068.

Lee, S. B. and Rhee, S. G. (1995). Significance of PIP2 hydrolysis and regulation of phospholipase C isozymes. *Curr. Opin. Cell Biol.* **7**, 183-189.

Leung, T., Manser, E., Tan, L. and Lim, L. (1995). A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J. Biol. Chem.* **270**, 29051-29054.

Lew, D. J. and Reed, S. I. (1995). Cell cycle control of morphogenesis in budding yeast. *Curr. Opin. Gen. Dev.* **5**, 17-23.

Madaule, P., Axel, R. and Myers, A. M. (1987). Characterization of two members of the *rho* gene family from the yeast *Saccharomyces cerevisiae*. *Proc. Nat. Acad. Sci. USA* **84**, 779-783.

Marks, J. and Hyams, J. S. (1985). Localization of F-actin through the cell division cycle of *Schizosaccharomyces pombe*. *Eur. J. Cell Biol.* **39**, 27-32.

Marks, J., Hagan, I. M. and Hyams, J. S. (1986). Growth polarity and cytokinesis in fission yeast: the role of the cytoskeleton. *J. Cell Sci. Suppl. 5*, 229-241.

Mata, J. and Nurse, P. (1997). tea1 and the microtubular cytoskeleton are important for generating global spatial order within the fission yeast cell. *Cell* **89**, 939-949.

Miller, P. J. and Johnson, D. I. (1994). Cdc42p GTPase is involved in controlling polarized growth in *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **14**, 1075-1083.

Mitchison, J. M. and Nurse, P. (1985). Growth in cell length in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **75**, 357-376.

Moon, A. L., Janmey, P. A., Louie, K. A. and Drubin, D. G. (1993). Cofilin is an essential component of the yeast cortical cytoskeleton. *J. Cell Biol.* **120**, 421-435.

Moreno, S., Klar, A. and Nurse, P. (1991). Molecular genetic analysis of the fission yeast *Schizosaccharomyces pombe*. *Meth. Enzymol.* **194**, 795-823.

Mulholland, J., Preuss, D., Moon, A., Wong, A., Drubin, D. G. and Botstein, D. (1994). Ultrastructure of the yeast actin cytoskeleton and its association with the plasma membrane. *J. Cell Biol.* **125**, 381-391.

Nakano, K. and Mabuchi, I. (1995). Isolation and sequencing of two cDNA clones encoding Rho proteins from the fission yeast *Schizosaccharomyces pombe*. *Gene* **155**, 119-122.

Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A. and Takai, Y. (1995). A downstream target of *RHO1* small GTP-binding protein is *PKC1*, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*. *EMBO J.* **14**, 5931-5938.

Nurse, P., Thuriaux, P. and Nasmyth, K. (1976). Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **146**, 167-178.

Ottilie, S., Miller, P. J., Johnson, D. I., Creasy, C. L., Sells, M. A., Bagrodia, S., forsburg, S. L. and Chernoff, J. (1995). Fission yeast *pak1+* encodes a protein kinase that interacts with Cdc42p and is involved in the control of cell polarity and mating. *EMBO J.* **14**, 5908-5919.

Prentice, H. L. (1992). High efficiency transformation of *Schizosaccharomyces pombe* by electroporation. *Nucl. Acids Res.* **20**, 621.

Qadota, H., Python, C. P., Inoue, S. B., Arisawa, M., Anraku, Y., Zheng, Y., Watanabe, T., Levin, D. E. and Ohya, Y. (1996). Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-β-glucan synthase. *Science* **272**, 279-281.

Ren, X. D., Bokoch, G. M., Traynor-Kaplan, A., Jenkins, G. H., Anderson, R. A. and Schwartz, M. A. (1996). Physical association of the small GTPase Rho with a 68 kDa phosphatidylinositol 4-phosphate 5-kinase in Swiss 3T3 cells. *Mol. Biol. Cell* **7**, 435-442.

Ribas, J. C., Díaz, M., Durán, A. and Pérez, P. (1991). Isolation and characterization of *Schizosaccharomyces pombe* mutants defective in cell wall β(1-3)-D-glucan. *J. Bacteriol*. **173**, 3456-3462.

Ridley, A. J. (1995). Rho-related proteins: actin cytoskeleton and cell cycle. *Curr. Opin. Genet. Dev.* **5**, 24-30.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.

Snell, V. and Nurse, P. (1993). Investigations into the control of cell form and polarity: the use of morphological mutants in fission yeast. *Development Suppl*. 289-299.

Toda, T., Shimanuki, M. and Yanagida, M. (1993). Two novel protein kinase C-related genes of fission yeast are essential for cell viability and implicated in cell shape control. *EMBO J.* **12**, 1987-1995.

Verde, F., Mata, J. and Nurse, P. (1995). Fission yeast cell morphogenesis: identification of new genes and analysis of their role during the cell cycle. *J. Cell Biol.* **131**, 1529-1538.

Welch, M. D., Holtzman, D. A. and Drubin, D. G. (1994). The yeast actin cytoskeleton. *Curr. Opin. Cell. Biol*. **6**, 110-119.

Watanabe, G., Saito, Y., Madaule, P., Ishizaki, T., Fujisawa, K., Morii, N., Mukai, H., Ono, Y., Kakizuka, A. and Narumiya, S. (1996). Protein kinase N (PKN) and PKN-related protein rhophilin as targets of small GTPase Rho. *Science* **271**, 645-648.

Yamamoto, M., Marui, N., Sakai, T., Morii, N., Kozaki, S., Ikai, K., Imamura, S. and Narumiya, S. (1993). ADP-Ribosylation of the rhoA gene product by botulinum C3 exoenzyme causes Swiss 3T3 cells to accumulate in the G_1 phase of the cell cycle. *Oncogene* **8**, 1449-1455.

Yamochi, W., Tanaka, K., Nonaka, H., Maeda, A., Musha, T. and Takai, Y. (1994). Growth site localization of Rho1p small GTP-binding protein and its involvement in bud formation in *Saccharomyces cerevisiae*. *J. Cell Biol*. **125**, 1077-1093.

Zheng, Y., Bender, A. and Cerione, R. A. (1995). Interaction among proteins involved in bud-site selection and bud-site assembly in *Saccharomyces cerevisiae. J. Biol. Chem*. **270**, 626-630.

(Received 16 April 1997 – Accepted 6 August 1997)